

Effect of inhaled fluticasone with and without salmeterol on airway inflammation in asthma

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Background: The clinical benefit of combining long-acting β_2 -agonists with inhaled corticosteroids rather than doubling the dose of corticosteroid has been well-documented. However, there are concerns that this might result in a masking of underlying airway inflammation.

Objective: The aim of this study was to test the hypothesis that the addition of the long-acting β_2 -agonist salmeterol (SALM) to a low dose of the inhaled corticosteroid fluticasone propionate (FP) has a steroid-sparing effect and does not result in a worsening of bronchial inflammation compared to doubling the dose of inhaled corticosteroid.

Methods: Fifty-six asthmatic subjects, previously not well-controlled on inhaled corticosteroids, were randomized to receive 3 months of treatment with inhaled FP 500 μ g twice a day (FP 1000) or FP 200 μ g twice a day plus SALM 50 μ g twice a day (FP 400 + SALM). Fluticasone propionate 200 μ g twice a day served as the control (FP400). Bronchial mucosal biopsy specimens, bronchial washings (BW), and bronchoalveolar lavage were obtained before and after treatment. The primary end points for the study were submucosal mast cell and eosinophil counts.

Results: There was a significant improvement in FEV₁ in the FP400 + SALM group compared to both the FP400 and FP1000 groups. This was accompanied by a significant improvement in peak expiratory flow in the FP400 + SALM group in both the morning and evening compared to the FP1000 group. There were no significant between treatment differences in the change in the number of submucosal mast cells or eosinophils. However, in the FP400 + SALM group there was a significant decrease in submucosal mast cells after 12 weeks of treatment. The addition of SALM to FP was not associated with any increases in airway inflammation in the biopsy specimens, bronchoalveolar lavage, or bronchial washings.

Conclusion: These findings confirm that addition of SALM to

FP has clinical benefits but does not mask or exacerbate airway inflammation and suggest that long-acting β_2 -adrenoceptor agonists might influence mast cell numbers. (J Allergy Clin Immunol 2003;112:72-8.)

Key words: Asthma, randomized controlled trial, bronchoscopy, fluticasone propionate, salmeterol, inflammation

Inhaled corticosteroids are the mainstay of asthma treatment. Their regular use results in the reduction in mast cell, eosinophil, and T-lymphocyte numbers and indices of cell activation in both bronchial biopsy specimens and bronchoalveolar lavage (BAL); these are paralleled by improved clinical indices of asthma and lung function.¹⁻⁴ The emphasis on prophylactic treatment with this class of drug has been central to national and international guidelines on asthma management, with recommendations that short-acting β_2 -adrenoceptor agonists should be used only as required to relieve breakthrough symptoms.⁵

Among the topical corticosteroids available, fluticasone propionate (FP) has been introduced on account of its increased efficacy and reduced systemic side effects. When administered on a regular basis to patients with active asthma, FP exhibits marked anti-inflammatory activity, as shown in both bronchial biopsy specimens and BAL,⁶⁻⁸ with low systemic absorption at low doses (less than 1000 μ g twice a day).⁹ For asthma that is inadequately controlled with low or moderate doses of inhaled corticosteroids, it has become accepted practice that the dose should be progressively increased to achieve disease control. However, because inhaled corticosteroids have a relatively flat dose-response curve,¹⁰ the beneficial effects gained on asthma might be accompanied by local or systemic side effects. This concern prompted the pioneer studies of Greening et al¹¹ and Woolcock et al,¹² who investigated the effect of adding the long-acting β_2 -agonist salmeterol (SALM), compared to the use of higher doses of inhaled beclomethasone dipropionate, in asthma patients with symptoms persisting despite existing inhaled corticosteroids. These and a series of related studies¹³⁻¹⁶ have clearly demonstrated that the addition of an inhaled long-acting β_2 -agonist to low or moderate doses of inhaled corticosteroids is more efficacious than doubling the dose of inhaled corticosteroids when assessed by pulmonary function and patient centered-outcome measures. A recent meta-

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Supported by a grant from Glaxo Wellcome R&D, United Kingdom.

Received for publication October 31, 2002; revised March 7, 2003; accepted for publication March 13, 2003.

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0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1518

Abbreviations used

BAL:	Bronchoalveolar lavage
BW:	Bronchial washing
FP:	Fluticasone propionate
ICAM-1:	Intercellular adhesion molecule 1
PEF:	Peak expiratory flow
PC ₂₀ :	Provocative concentration required to induce a 20% decrease in FEV ₁
SALM:	Salmeterol
VCAM-1:	Vascular cell adhesion molecule 1

analysis¹⁷ of 6 of these trials involving 3685 patients with moderately severe disease consistently showed an added benefit of SALM over increasing inhaled corticosteroids in patients. The FACET study¹⁸ has also shown similar effects with formoterol, including a preferential reduction in asthma exacerbations over that achieved by doubling the dose of inhaled corticosteroids.

The mechanism(s) underlying the benefit of adding long-acting β_2 -adrenoceptor agonists to inhaled corticosteroids in asthma management is not known. Both formoterol and SALM are powerful bronchodilators and functional antagonists of airway smooth muscle and undoubtedly exert some of their clinical benefit in moderate to severe asthma through these mechanisms. On the basis of this, there has been some concern that adding a long-acting inhaled β_2 -agonist to inhaled corticosteroids in situations in which the latter are inadequate to optimally control the disease might result in masking of airway inflammation even though symptoms might diminish. The study of Li et al¹⁶ demonstrated that the addition of 50 μ g SALM twice a day to 500 μ g beclomethasone dipropionate or budesonide twice a day did not result in any worsening in airway inflammation. In fact, a decrease in EG1+ cells was observed with combination treatment. In another study formoterol¹⁹ has been shown to exhibit some anti-inflammatory effect on mast cells when assessed in bronchial biopsy specimens. More recently formoterol in combination with budesonide has been shown to inhibit proliferation of primary bronchial smooth muscle cells²⁰ *in vitro*.

The aim of this present study was to test the hypothesis that the addition of the long-acting β_2 -agonist SALM to a low dose of the inhaled corticosteroid FP has a steroid-sparing effect and does not result in a worsening of bronchial inflammation compared to doubling the dose of inhaled corticosteroid. This was a 12-week, parallel group, randomized controlled trial, in which the efficacy of each treatment on clinical measures of airway caliber, bronchial hyperresponsiveness, and airway inflammation in mucosal biopsy specimens, bronchial washing (BW), and BAL obtained by fiberoptic bronchoscopy was assessed. Submucosal eosinophil and mast cell counts were the primary end points. A power calculation was made with data from a previous study, which compared the percentage change in submucosal mast cell numbers before and after formoterol, another long-acting

β_2 -agonist, and before and after placebo.¹⁹ In this study the median between group difference was 42%. To detect this same difference between groups in this present study it was calculated that 20 patients per group would give a power of 80% with a 2-tailed significance of 5%.

METHODS

Study design

This was a randomized double-blind, parallel group, control study, which comprised a 2- to 4-week run-in period followed by a 12-week treatment period conducted outside the pollen season. During the run-in period and after treatment, a methacholine bronchial provocation test and fiberoptic bronchoscopy were undertaken. After the first bronchoscopy the subjects were randomized to receive FP 200 μ g twice a day (FP 400), 500 μ g twice a day (FP 1000), or 200 μ g twice a day together with SALM 50 μ g twice a day (FP 400 + SALM) administered by the dry powder Discus/Accuhaler inhaler (GlaxoSmithKline R&D, Uxbridge, United Kingdom) plus salbutamol via the Diskhaler inhaler (200 μ g per inhalation) or metered dose inhaler (GlaxoSmithKline) (100 μ g per puff) as required for symptom relief. This study was approved by the Ethics Committee of Umeå University (project 95-154) and by the Regional Ethics Committee in Trondheim (project 10-96). All subjects gave written informed consent.

Subjects and clinical assessments

To be included in the study the subjects had to have been free of respiratory tract infections within the preceding 4 weeks and have a clinical need for extension of treatment on the basis of both symptoms and pulmonary function during the last 2 weeks of the run-in period despite the use of budesonide 800 to 1200 μ g per day or FP 400 to 500 μ g. Full details of inclusion criteria are shown in Fig 1.

Asthma symptoms, peak expiratory flow (PEF), and inhalations of salbutamol were recorded twice daily on diary cards. Bronchial responsiveness was assessed according to Juniper et al,²¹ with salbutamol and trial medication being withheld for at least 6 and 12 hours, respectively, before methacholine challenge. Exacerbations were treated with prednisolone 30 mg daily for 5 days. If not resolved after 5 days or less than 4 weeks before the second bronchoscopy, the patient was withdrawn from the study.

Bronchoscopy

Bronchoscopy was conducted by using standard procedures conforming to the National Heart, Lung, and Blood Institute^{22,23} guidelines. At each bronchoscopy, 4 to 6 mucosal biopsy specimens were taken from 1 lung. BW and BAL were sequentially performed by instillation of 2 $\times$ 20 mL and 3 $\times$ 60 mL of sterile phosphate-buffered saline, pH 7.3, respectively, in the contralateral lung and processed as previously described.¹⁹

Immunohistochemistry

Biopsy specimens were processed into glycol methacrylate for immunohistochemical analysis. Immunohistochemical analysis with the streptavidin biotin-peroxidase technique, as previously described,²⁴ was divided into 3 phases: (1) inflammatory cells including mast cells and eosinophils, the study primary end points; (2) adhesion molecules and ligands; and (3) cytokines. Phases 2 and 3 were only undertaken to investigate the mechanisms of action of the therapies if significant results were obtained in phase 1. Details of the monoclonal antibodies are listed in Table I. Positively stained nucleated cells were counted in the lamina propria, area was measured with the assistance of computerized image analysis, and the number of cells per square millimeter was calculated.

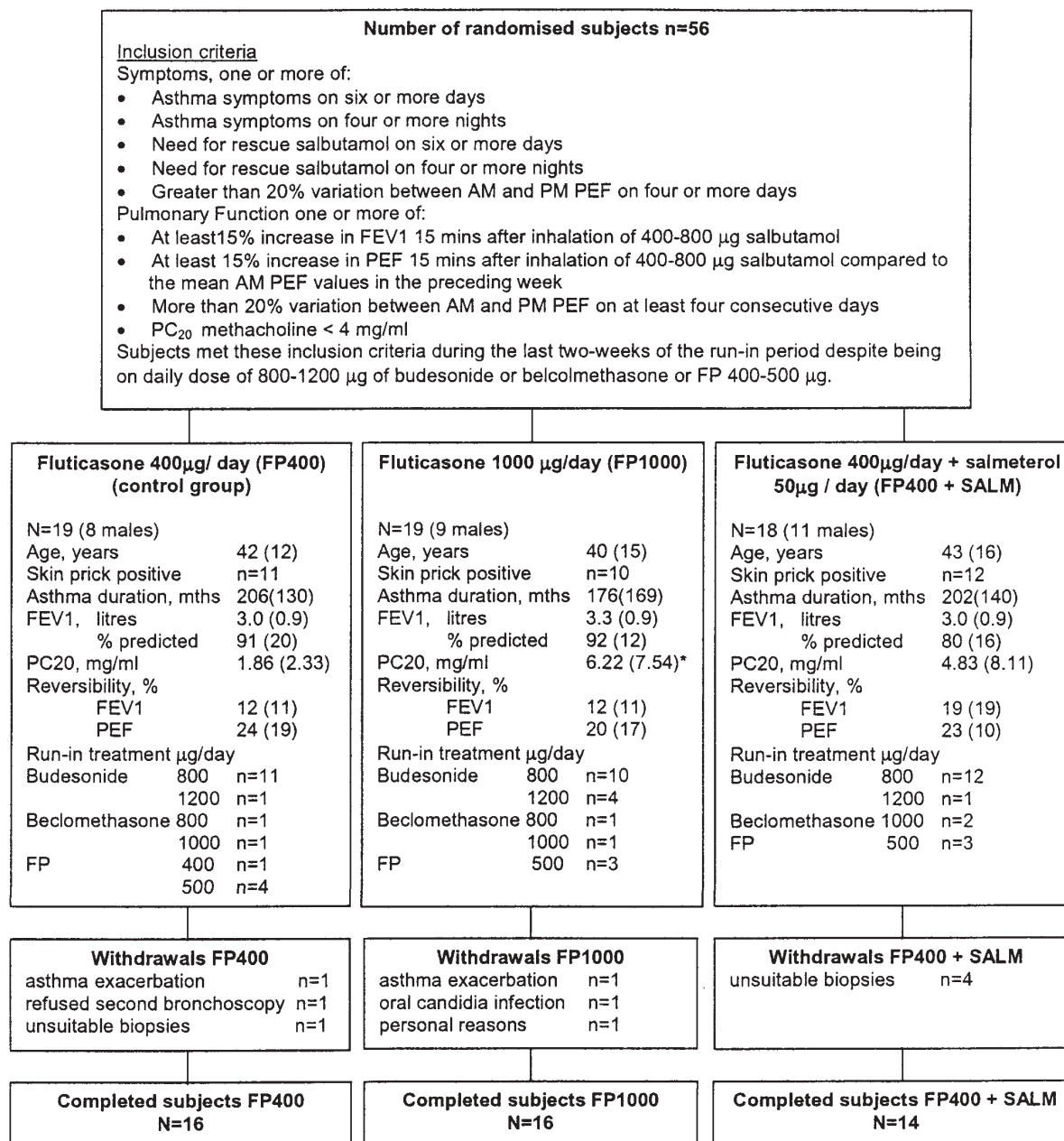


FIG 1. Flow diagram indicating the total number of subjects randomized and the study inclusion criteria. The clinical characteristics for each treatment are summarized; the withdrawals within each group and total number of completed subjects are also given. All data are mean (± SE). **P* < .05 compared with FP 400.

BAL and BW

The fluid from the BW and BAL was kept on ice during transport to the laboratory. After filtration through a nylon filter with a pore diameter of 100 µm (Syntab Product AB, Tranås, Sweden), the fluid was centrifuged at 400g for 15 minutes at 4°C. The cells were immediately processed, and cytopins were prepared and stained by May Grunwald Giemsa method for differential counting and by acid toluidine blue for enumeration of mast cells. The supernatant was frozen at -80°C until analysis.

Markers of inflammation, inflammatory cell activation, and fibroblastic activity were measured in the supernatant of the BW and BAL fluid. Albumin was assayed by a fluorometric method

(Beckman Array Protein System; Beckman Instruments Inc, Fullerton, Calif). The concentrations of eosinophil cationic protein and methyl histamine were measured with commercial radioimmunoassay kits (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), and hyaluronan and fibronectin were determined by a radiometric assay (Pharmacia HA test, detection limit of 5 µg/L) and ELISA (detection limit of 10 µg/L), respectively. Soluble intercellular adhesion molecule-1 (ICAM-1) was measured with commercial ELISA kits (R&D Systems, Minneapolis, Minn).

Subtyping and the activation status of T lymphocytes in the BAL fluid were determined by flow cytometry with the FACSCAN flow cytometer (Becton-Dickinson Immunocytometry Systems, San

TABLE I. Monoclonal antibodies

Antibody	Epitope stained	Dilution	Source
Phase I: Inflammatory cells			
AA1	Mast cells	1:1000	Dako, Cambridge, UK
EG2	Eosinophils	1:500	Pharmacia & Upjohn, Milton Keynes, UK
NOE	Neutrophils	1:1000	Dako, Cambridge, UK
CD3	T lymphocytes	1:100	Dako, Cambridge, UK
CD4	T-helper cells	1:10	Becton Dickinson, Oxford, UK
CD8	T-suppressor cells	1:100	Dako, Cambridge, UK
CD25	Activated T cells	1:50	Dako, Cambridge, UK
Phase II: Adhesion molecules and ligands			
CD62P	P selectin	1:20	Becman Coulter, Luton, UK
CD62E	E selectin	1:15	Cymbus Biotechnology, Southampton, UK
CD54	ICAM-1	1:600	gift, R Rothlein, Boehringer Ingelheim, Ridgefield, Conn
CD106	VCAM-1	1:20	Serotec, Oxford, UK
CD11a	LFA-1	1:2000	Cymbus Biotechnology, Southampton, UK
CDw49d	VLA-4	1:10	Cymbus Biotechnology, Southampton, UK
Phase III: Adhesion molecules and ligands			
3H4	Secreted IL-4	1:50	Immunokontakt, Frankfurt, Germany
4D9	IL-4	1:50	Immunikontakt, Frankfurt, Germany
MAB7	IL-5	1:100	gift, P Hissey, Glaxo Wellcome, Stevenage, UK
52B83	TNF- α	1:100	gift, Celltech, Slough, UK

UK, United Kingdom.

Monoclonal antibodies used for immunohistochemical staining.

Jose, Calif). Cell pellets obtained by centrifugation of 1.5- to 2-mL aliquots of BAL fluid at 2500g for 5 minutes at 4°C were incubated with mouse monoclonal antibodies (Becton Dickinson Immunocytometry Systems; DAKO, Copenhagen, Denmark; Ortho Diagnostic System Inc, Raritan, NJ) to identify surface markers of mature T cells (CD3), T-helper cells (CD4), T-suppressor cells (CD8), natural killer cells (CD16+56), ICAM-1 positive cells, and lymphocyte activation markers HLA-DR and IL-2 receptor (CD25).

Statistical analyses

For the primary study end points, submucosal mast cell and eosinophil counts, both between group and within group comparisons were made. For all other data the analyses were exploratory, and only between group comparisons were made. The data for PEF, symptom scores, use of rescue medication, immunohistochemistry, BW and BAL measurements were not normally distributed and were therefore analyzed with nonparametric statistics. Between group differences were initially analyzed by the Kruskal-Wallis ANOVA, and if significant at the 5% level the Mann-Whitney *U* test was used to determine between which groups these differences occurred. Mann-Whitney *U* results were corrected for multiple testing by using the Holm's step-down correction method.²⁵ Within group comparisons for the primary end points were made by using the Wilcoxon signed rank test; a *P* value of less than .05 was considered significant. PC₂₀ methacholine was logarithmically transformed. Between group differences for PC₂₀ and FEV₁ were initially analyzed by using analysis of covariance, and if this was significant, the Student *t* test was used to determine between which groups these differences occurred. A Bonferroni correction was used for multiple testing of parametric data.

RESULTS

Baseline characteristics of the subjects in the 3 groups were evenly distributed with respect to gender, asthma duration, measures of pulmonary function, and reversibility. For the baseline PC₂₀ there was a significant

difference (*P* < .05) between the subjects randomized to FP 1000 when compared with FP400 (Fig 1).

Fifty-six subjects were randomized, of which 51 underwent both bronchoscopies. Of the 5 who failed to complete the study, 2 were withdrawn because of an asthma exacerbation (FP 1000 and FP 400), one subject was withdrawn because of an oral candidal infection (FP1000), one withdrew for personal reasons (FP 1000), and the last subject did not want to undergo repeat bronchoscopy (FP 400). This is summarized in Fig 1.

Clinical indices

Five subjects in the FP 400 group, 2 in the FP 1000 group, and 1 in the FP 400 + SALM group had exacerbations of their asthma during the 12-week treatment period. Two subjects (FP 1000 and FP 400) were withdrawn, but the exacerbations in the others resolved after a 5-day course of prednisolone.

Several significant improvements in clinical indices were observed (analysis of covariance: FEV₁, *P* = .002; Kruskal-Wallis: PEF AM, *P* = .013; PEF PM, *P* = .018). There was a significant improvement in FEV₁ after treatment with FP 400 + SALM from a mean of 3.0 to 3.4 L compared to treatment with FP 400 (3.0 to 3.1 L, *P* = .003) and FP 1000 (3.3 to 3.3 L, *P* = .001) (Fig 2, A). Morning and evening PEF improved significantly during the treatment period in the FP 400 + SALM group from a median of 441 to 478 L/min in the morning and 467 to 494 L/min in the evening, compared to a change from 456 to 473 L/min in the morning in the FP 1000 group (*P* = .001) and a change from 471 to 474 L/min in the evening in the FP 1000 group (*P* = .016) (Fig 2, B and C). There were no other significant changes in clinical parameters (data not shown).

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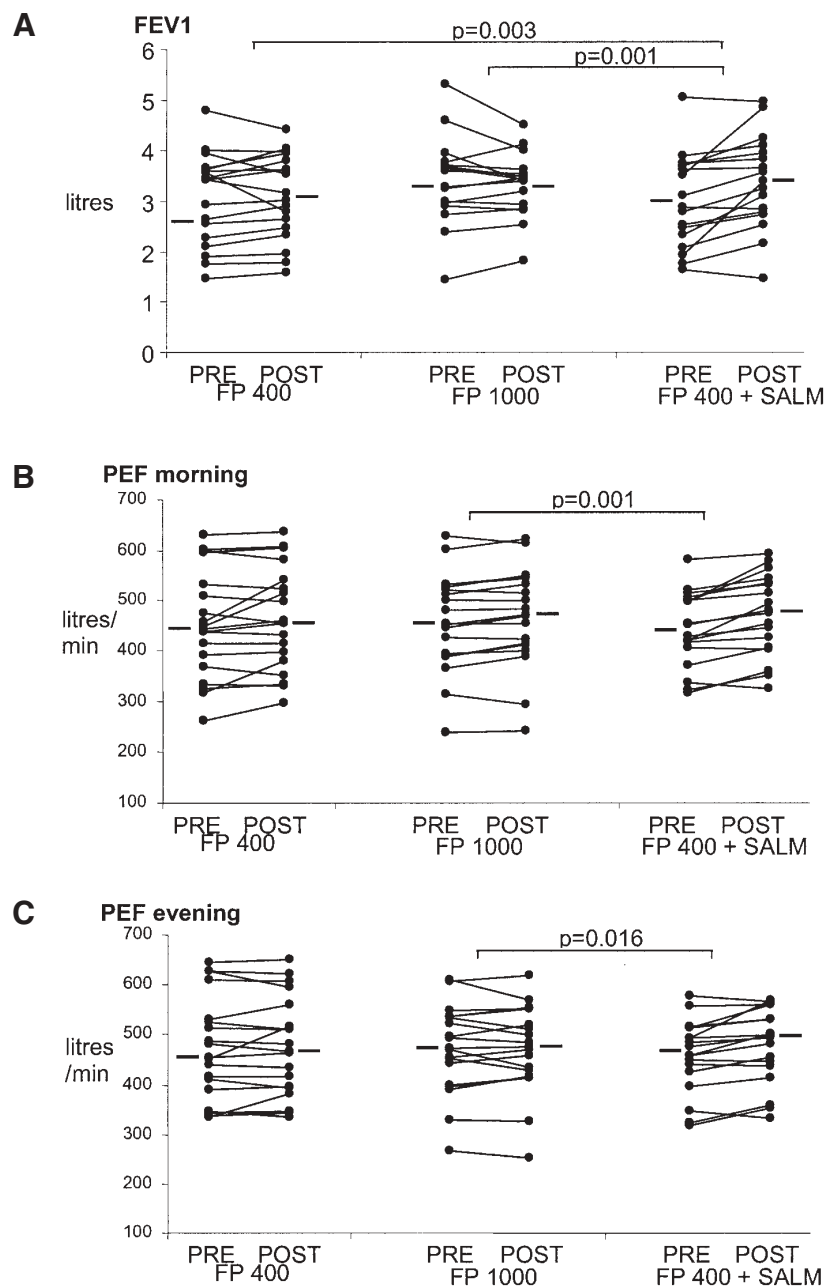


FIG 2. Graphs of clinical data depicting (A) FEV₁, (B) morning PEF, and (C) evening PEF before and after each treatment. Significant differences are indicated.

Bronchial biopsies

Of the 51 subjects who underwent both bronchoscopies, paired analyzable biopsy specimens were obtained from 46 subjects (Fig 1).

Inflammatory cells (phase 1). For the primary end points of this study, mast cell and eosinophil numbers, there were no significant between group differences at the 5% level. However, within group analysis showed that the combination of FP 400 + SALM reduced the number of

mast cells from a median of 9.9 cells/mm² to 7.3 cells/mm² ($P = .04$) during the treatment period (Fig 3).

There were no other significant between treatment changes in inflammatory cell numbers (data not shown). Because significant within group differences were observed for mast cell numbers, biopsy specimens were further analyzed for the expression of adhesion molecules and cytokines.

Adhesion molecules (phase 2) and cytokines (phase 3). There were no significant between group differences in

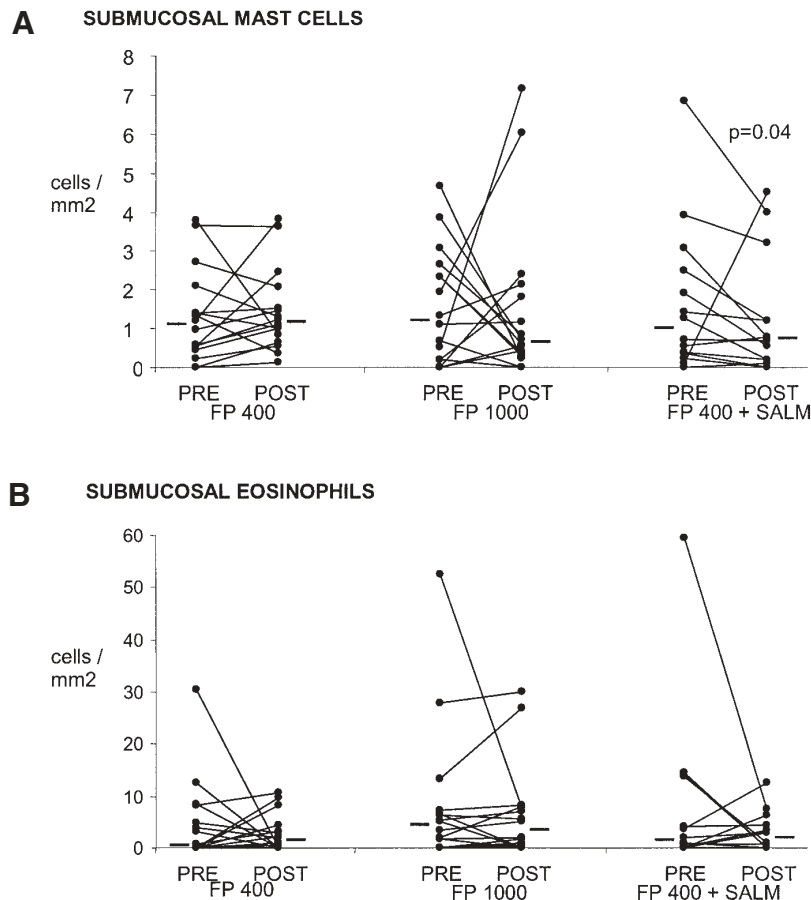


FIG 3. Graph showing the number of (A) mast cells and (B) eosinophils in the submucosa. Significant changes are shown. These were the primary study end points.

the expression of any of the adhesion molecules or cytokines studied (data not shown).

BW and BAL

There were no significant between group differences in any of the cells or mediators measured in either the BW or BAL (data not shown).

DISCUSSION

The present study was designed to test the hypothesis that the addition of the long-acting β_2 -agonist SALM to a low dose of the inhaled corticosteroid FP has a steroid-sparing effect and does not result in a worsening of bronchial inflammation compared to doubling the dose of inhaled corticosteroid.

By using objective measures of pulmonary function the present study has confirmed previous observations¹¹⁻¹⁶ that the addition of the long-acting β_2 -agonist SALM to a low dose of FP in symptomatic asthmatic patients results in superior asthma control when compared to increasing the dose of inhaled corticosteroids by more than 2-fold. The combination therapy during a period of 12 weeks resulted in a superior clinical response when

assessed as FEV₁ and PEF. These findings substantiate the large amount of data supporting the view that, in symptomatic patients taking regular inhaled corticosteroids, the addition of a long-acting inhaled β_2 -agonist is superior to increasing the dose of corticosteroid in bringing asthma under control.¹¹⁻¹⁸ Although the mean PC₂₀ appeared to improve with all treatments during the study period, there were no significant differences between the 3 treatment groups. The lack of between group differences is not unexpected, because Woolcock et al¹² have suggested that maximal achievable improvement in PC₂₀ is attained with doses of 400 to 500 μ g of corticosteroid.

There has been some concern that although addition of a longacting inhaled β_2 -agonist to inhaled corticosteroids might improve clinical indices of disease, they might result in a masking of airway inflammation. In this study, as in that of Li et al,¹⁶ we have demonstrated that the addition of the SALM therapy to that with FP does not result in a worsening of inflammation in biopsy specimens, BAL or BW. Indeed, we observed a decrease in submucosal mast cells and Li et al¹⁶ a decrease in eosinophil numbers, suggesting that combination therapy might have some additional anti-inflammatory effects to

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those observed when the treatments are used singularly. The mechanism and biologic relevance of these effects in the absence of changes in mast or eosinophil mediators are unknown and clearly warrant further research. More recently Roth et al²⁰ have demonstrated in vitro that budesonide in combination with formoterol leads to an inhibition of bronchial smooth muscle proliferation. Whether a similar inhibition of proliferation occurs in vivo needs to be investigated but was outside the remit of this current study.

The anti-inflammatory effects of inhaled corticosteroids in corticosteroid naïve subjects are well-documented^{1-4,6-8} and include a decrease in the number of eosinophils in the airway mucosa. This is again substantiated in the present study with the finding of baseline mucosal eosinophil counts within the normal range, with only 7 of 56 subjects having more than 10 eosinophils per mm² submucosa. All subjects had been treated with inhaled corticosteroids (budesonide or beclomethasone 800 to 1200 µg or FP 400 to 500 µg daily) for at least 4 weeks before randomization. The lack of an effect after randomization on eosinophil mucosal counts and BAL levels of eosinophil cationic protein most likely reflects the low numbers of eosinophils present at baseline. The study of Li et al¹⁶ did observe a further decrease in eosinophil numbers with the addition of SALM; however, the subjects were on much lower doses of steroids than those in our present study.

In conclusion, no increase in mucosal inflammation has been observed on the addition of SALM to low dose inhaled FP in patients requiring additional treatment for persistent disease. On the contrary, it is suggested that there might be a decrease in the inflammatory response. Whether this can account for some of the efficacy of combined therapy clearly warrants further research. The combined effect of a low dose corticosteroid and long-acting β_2 -agonists on clinical and inflammatory parameters further promotes the benefits of this class of therapy.

We would like to thank Kevin Kane (GlaxoSmithKline) for undertaking the statistical analysis of the clinical data.

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